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ONTARIO RESEARCH ENHANCEMENT PROGRAM (O R E P)

Final Report

Development of Transgenic Soybean with Cyst Nematode Resistance

REPORT No. OREP - 1999/11

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Canada

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FORWARD

The Ontario Research Enhancement Program (OREP) is a $4-million two-year federal research initiative that is administered by the Research Branch of Agriculture and Agri-Food Canada (AAFC), with input from the agriculture and agri-food sector, universities and the province. Research focuses on two areas identified by the sector as:

1. Priorities responding to consumer demand for higher quality products; and
2. Ensuring crop-production management systems are environmentally sustainable.

Biotechnology is one of the areas to be explored. Ontario's agricultural production is based primarily on growing diversified crops using intensive production practices, but long-term viability is linked to the development of sustainable crop production management systems.

There are also opportunities to enhance the economic contribution of Ontario's agriculture and agri-food sector by adding value to the diversified primary commodities produced in the province. The Program is expected to be of particular interest to the corn, soybean, greenhouse, fruit and vegetable sectors and the emphasis will be on projects focussing on food quality improvement and sustainable crop production management such as:

1. The development of value-added animal and crop food products and ingredients that meet domestic and export market demands, with a particular emphasis on quality issues such as processing, packaging, shelf life, new product and ingredient development, high-value alternative uses, probiotics and functional foods;
2. Biotechnologies to reduce environmental pressures related to crop protection practices; and,
3. Integrated crop protection and production practices for both field and greenhouse crops.

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Executive Summary

Soybean production in Ontario currently suffers annual losses of $10 M due to the soybean cyst nematode (SCN) (Tenuta, 1996; Olechowski, 1997), and the potential for further damage is great (Labaj and Mallet, 1996). No effective method exists for eradicating SCN (Olechowski, 1997), and the use of resistant soybean cultivars is the most economical means of control. Many pathogenic races of SCN exist, with at least four in Ontario, and conventionally-bred cultivars of resistant soybean last usually less than three years before resistance is broken (Nieblack, 1999). There are few SCN-resistant cultivars suitable for southern regions of Canada, and where they are available, they are generally not as productive as the best susceptible cultivars on non-infested land (Ontario Oil and Protein Seed Crop Committee, 2001). The introduction of SCN resistance into a locally-adapted soybean cultivar using conventional breeding is time consuming. An alternative and more rapid approach to solving the SCN problem is the use of biotechnology.

In the present project, a nematode-resistance gene from wild beet, as well as a homologue of that gene from *Arabidopsis*, were introduced into one of two soybean varieties by transformation biotechnology. Overall, 502 transgenic soybean plants were produced, and 4098 of their offspring were subjected to a blind screening for cyst nematode resistance in an infested field in Ruthven, Ontario, in the summer of 1999. First-generation seeds after gene transformation were collected from 23 transgenic lines which showed potential SCN resistance; among these, 15 were made with the wild beet gene, whereas eight were made with the *Arabidopsis* gene. One of these transgenic lines was selected as SCN resistant in two different trials of the field test, and thus it has the highest priority among the produced lines for development into a novel SCN-resistant cultivar.

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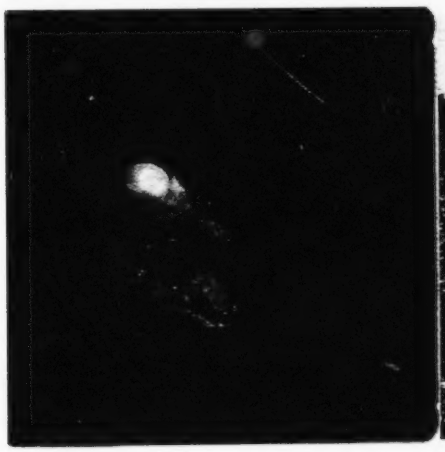
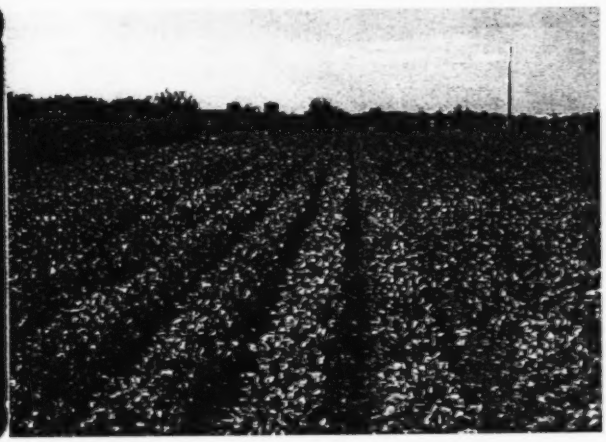
A. Introduction

Although nematodes comprise a large phylum of animals, three genera cause the most agricultural damage worldwide. Two are cyst nematodes: *Heterodera* and *Globodera*. A third, *Meloidogyne,* are root-knot nematodes. The SCN, *Heterodera glycines*, was first noticed in North Carolina in 1954 (Winstead et al. 1955). It is already the most important economic pathogen of the soybean crop in the United States (Williamson and Hussey, 1996), and is likely also for the crop in Ontario, with at least $10 M being the predicted loss for the provincial crop in 1998 (based on approximately 25 % overall loss in yield, 150,000 currently infested acres (A. Tenuta, personal. communication), and $300 value per acre (OMAFRA, 2000)).



Immature cysts on infected soybean



Commercial soybean filed with evidence of foliar yellowing often associated with SCN damage

Chemical treatments such as soil fumigation are not currently registered for agricultural use in Canada against the SCN (Olechowski, 1997). Furthermore, eradication of an established field population of SCN is not possible because the cyst provides protection to the enclosed eggs, and egg hatching is extended over several years. The tobacco industry depends upon soil fumigation to control nematodes; in 1993 for example, greater than 1.5 million litres of soil fumigant formulations were applied to tobacco crops in Ontario (OMAFRA, 1994). Growers of other high value crops, such as ginseng and tomato, may even apply fumigants as a precaution without establishing a need for their use. Such

treatments are economically prohibitive for the soybean industry, with fumigation costs reaching approximately $200 per acre (based on the use of Telone- and Vorlex-brand formulations in the tobacco industry; OMAFRA, 1994).

Chemical treatments involving synthetic nematicidal compounds are primarily nerve poisons (organophosphorus compounds such as Mocap, and methylcarbamates such as Temik; Miller and Chalmers, 1987; Casida, 1993). The active ingredients of all present fumigants are either carcinogens (eg. methylisothiocyanate) or chlorinated hydrocarbons (eg. 1,3-dichloropropene and chloropicrin; Noling and Becker, 1994) that contribute to the deterioration of the ozone layer. Although chemical treatments for SCN may become available in Canada, it is desirable for economic, safety and environmental reasons to develop alternative methods that reduce the needs for these treatments.

The use of resistant soybean cultivars such as Pickett, Custer and Forest which are derived from 'Peking', PI88788, PI90763, PI437654 and PI209332, is the most economical practice for controlling cyst nematode. These cultivars are typically resistant to only single nematode races (i.e. races 1, 3, 4, 9, 14) (Wrather et al., 1984; Anand et al., 1988; Anand, 1991; Riggs and Wrather, 1992; Kinloch 1998). One cultivar, Hartwig, is resistant to all known races of SCN. Monoculture of resistant cultivars is discouraged because of the pressure that such a practice exerts for selection of resistance-breaking races within heterogeneous SCN populations (Young, 1994; Nieblack, 1999). There are few SCN-resistant cultivars for southern Canada maturity groups (i.e. 1, 0 and 00), and where they are available, they are generally not as productive as the best susceptible cultivars on non-infested land. In 1998-2000 field trials conducted on infested lands in southwestern Ontario, the eleven SCN-resistant cultivars tested gave average yields of 30 % greater than the best susceptible cultivars (Ontario Oil and Protein Seed Crop Committee 2001). Multiple race-resistant cultivars are only now beginning to appear in soybean breeding programs.

Classical genetic studies demonstrated that inheritance of SCN resistance is complex and controlled by multiple genes, both dominant and recessive (Anand, 1994; Concibido et al., 1997). The addition of a single nematode resistance trait to a high yielding Ontario-adapted soybean cultivar would increase yields in nematode-infested areas; however, a classical breeding program aimed at this goal is time consuming. It takes eight to ten years to introduce a simple trait into a cultivar that is adapted to a particular locality, and cyst nematode resistance in soybeans is not a simple trait.

An alternative and more rapid approach to solving the SCN problem is the use of biotechnology. Whole-genome sequencing initiatives and positional gene cloning efforts have provided a number of candidate genes, primarily for use in medical applications, but also for use in agriculture (Strauss and Falkow, 1997). In the past few years, a number of genes that confer plant resistance to pathogens such as bacteria, viruses and fungi, have been identified (Moffatt, 1994). Recently, a gene from *Beta procumbens*, a wild relative of the sugar beet *B. vulgaris*, has been demonstrated to provide resistance to the beet cyst nematode (Cai et al., 1997). This newly discovered gene, *Hs1*$^{pro\text{-}1}$, is the first resistance gene against nematodes to be cloned and sequenced (Moffatt, 1997).

The beet cyst nematode, *H. schachtii*, against which the *Hs1*$^{pro\text{-}1}$ gene provides resistance, is very closely related to *H. glycines*. Interspecific hybridizations between *H. schachtii* and *H. glycines* are fertile, and the cross can be successfully taken through to the F_2 generation (Potter and Fox, 1965). The lack of a reproductive barrier between these two species of cyst nematode demonstrates an extremely similar genetic relatedness, and although different plant hosts are parasitized, the molecular mechanisms employed are likely similar. Thus, the mechanism of resistance provided by the *Hs1*$^{pro\text{-}1}$ gene against *H. schachtii* might also be effective against *H. glycines*, and as it would be a novel resistance for soybean against *H. glycines* this resistance could be effective against all races of SCN.

Although the predicted amino acid sequence of the protein encoded by *Hs1*$^{pro\text{-}1}$ provides clues about its function in the plant, the mechanism of nematode resistance is not known. The predicted polypeptide can be dissected into different subdomains, the most notable of which include a putative signal peptide (may target protein to cytoplasmic membrane), a leucine-rich domain (potential for protein-protein interactions), and a hydrophobic stretch (potential transmembrane segment). A potential mechanism involves a signaling pathway among plant (and possibly pathogen-encoded) proteins possessing sequences of similar leucine-rich repeats. The finding (Cai et al., 1997) that an *Arabidopsis* gene, EST 230M13T7, encodes a protein with 52% amino acid sequence identity (and 68% similarity; this report) indicates that the genomes of plants other than *B. procumbens* are likely to encode similar proteins. Therefore, successful interactions among proteins encoded by a transgenic host genome, such as soybean, with a protein encoded by an engineered *Hs1*$^{pro\text{-}1}$ transgene is predicted. Support for this comes from preliminary experiments in which the *Hs1*$^{pro\text{-}1}$ gene was introduced into different plant species (C. Jung, personal communication), demonstrating that the protein encoded by the transgene functions in the cells of different

plants. Thus, incorporation of the *Hs1*$^{pro\text{-}1}$ gene into soybean represents a promising strategy for the development of SCN-resistant cultivars.

At the outset of this project, only a portion of the *Arabidopsis* gene sequence was known; however, its entire sequence, including its promoter, was made public in the *Arabidopsis* GenBank sequence database at the end of 1997. The protein product of the *Hs1*$^{tha\text{-}1}$ gene has an unknown function, but it has approximately 60% overall amino acid sequence identity to the *Hs1*$^{pro\text{-}1}$ gene product. Therefore, its mode of action was predicted to be similar to the *Hs1*$^{pro\text{-}1}$ gene product.

In order to express these two genes in a transgenic system, a strong constitutive promoter was chosen (Ni et al., 1995). Thus, the potential nematode resistance traits from constructs involving the super promoter should be expressed throughout the plant during all developmental stages. The *Hs1*$^{pro\text{-}1}$ gene product, expressed with another strong constitutive promoter (CaMV 35S), was genetically stable (C. Jung, personal communication). The *Hs1*$^{tha\text{-}1}$ gene product was expected to behave in the same way.

We also chose to study the native *Hs1*$^{tha\text{-}1}$ promoter, primarily because of its ready availability. Tissues expressing the *Hs1*$^{tha\text{-}1}$ gene product from the construct containing the *Hs1*$^{tha\text{-}1}$ native promoter are unknown as this promoter has not yet been studied. Also, the developmental stage(s) at which the *Hs1*$^{tha\text{-}1}$ native promoter functions are not known.

The objective of this proposal, to test the hypothesis that the gene for cyst-nematode resistance in beet confers similar protection on soybean, is aimed at producing locally-adapted soybean cultivars that are nematode resistant in a relatively quick period of time. This is consistent with one of the OREP objectives: the development of soybean cultivars that would allow for a sustainable resistance to SCN. These genes may also have potential for use in the development of nematode resistance in other crop species. Thus, biotechnology may contribute to a reduction in environmental pressures associated with current agronomic and food production practices.

B. Objectives/Activity Milestones

The overall objective of this project was to test the hypothesis that the gene for beet cyst nematode resistance confers similar protection on soybean. The activity milestones included:

1. Researchers at the University of Guelph were to produce chimeric, nematode resistance genes in vectors suitable for soybean transformation.

2. Researchers at the ECORC (See Section L) were to use these chimeric gene constructions to produce transgenic soybean plants, using a cultivar that was closely related to soybean cultivars propagated in Ontario, and were to collect seed from these transgenic plants.

3. Researchers at the GPCRC were to test the progeny of these transgenic plants for nematode resistance.

4. Researchers at Guelph were to produce an antibody for the protein product of the *Hs1*$^{pro\text{-}1}$ gene for use in determining the relative levels of that gene product in SCN-resistant transgenic soybean plants.

C. Materials and Methods

1. Plasmid DNA constructions

The original version of the *Hs1*$^{pro-1}$ gene, provided by Dr. C. Jung of the University of Kiel, Germany, to the Shelp laboratory, after the signing of a materials transfer agreement, is a 1300-bp cDNA in the vector pGEM-T. This coding sequence, along with its native transcriptional terminator sequence, was placed behind the (*ocs*-UAS)$_3$ (*mas*-UAS/*mas*-P) "superpromoter", provided by Dr. S. Gelvin of Purdue University, West Lafayette, IN, under another signed materials agreement. This promoter functions in all plant cell types tested and is several-fold stronger than the CaMV 35S promoter (Ni et al., 1995). This superpromoter-*Hs1*$^{pro-1}$ fusion was placed in the plant transformation vector pFF19G (Timmermans et al., 1990) in front of the cauliflower mosaic virus (CaMV) 35S transcript terminator and sent to the Simmonds laboratory at the ECORC as our plasmid number 52. Another version of this plasmid, lacking the 35S terminator, was subsequently fabricated as our plasmid number 79 and sent to the ECORC. A negative control plasmid, which was made as our plasmid number 53 by inserting a premature stop codon at amino acid position 45 in the coding sequence using the Klenow fragment of DNA polymerase I to fill-in a unique *Eco*RI site, was also sent to the ECORC.

An *A. thaliana* homologue, which we called *Hs1*$^{tha-1}$, was found in the GenBank database and acquired as a bacterial artificial chromosome (BAC) clone, numbered T28M2, from the *Arabidopsis* Biological Resource Center (Columbus, OH). The native promoter (1.9 kbp), coding sequence (1.4 kbp), and transcriptional terminator (0.7 kbp) for this gene was removed from the BAC as a 4.0 kbp *Spe*I-*Nco*I fragment and placed in pGEMT (Promega Corp., Madison, WI) to create our plasmid number 71. The *Hs1*$^{tha-1}$ coding and terminator sequence was also placed behind the Gelvin superpromoter and placed in pBluescript (pBS-SK, Stratagene Ltd., La Jolla, CA) to create our plasmid number 74. Both of these plasmids were sent to the Simmonds laboratory at the ECORC.

2. Soybean transformations

Test material was transformed at the ECORC. The five plasmid constructs (#s 52, 53, 71, 74 and 79) were introduced into soybean cultivar X5, which is closely related to the Ontario cultivar AC Colibri, via particle gun bombardment and regeneration (Rogers et al., 1986; Timmermans et al., 1990). Plasmid number 74 was also introduced into the cultivar

AC Orford via bombardment and regeneration. These cultivars, which are adapted for growth in the northern and eastern parts of the Ontario soybean farming region, were chosen for this project as they are easily transformed and regenerated, and easily crossed with other soybean cultivars. The original transformed plants (T_0 generation) were tested for presence of the transgene (Southern analysis) using a fragment of the *Hs1*$^{pro-1}$ or *Hs1*$^{tha-1}$ coding sequence as a probe. Transgenic plants were allowed to set T_1 seeds, which were collected and labelled at the ECORC for blind testing.

3. SCN-resistance field test

T_1 plants, containing either the *Hs1*$^{pro-1}$ or *Hs1*$^{tha-1}$ genes, were screened for SCN resistance at a nematode-infested site near Harrow, Ontario. The trial site was located at the John Klassen farm in Ruthven, 8 kilometres west of Leamington on Queens Highway 3 in Essex County. The area, which is part of Agriculture & Agri-Food Canada's SCN experimental plots, is considered to have medium to high infestation (i.e. 2000-6000 eggs per 100 g soil) of races three and six. Surrounding fields contained corn and soybeans that were four metres from the trial border. The T_1 plants were compared to the susceptible control cultivars ("checks"), Kenwood 94 and S20-20, and to the cultivars, AC Orford and X5, from which the transgenic plants were derived.

Seeds from individual lines were planted in rows, with 60 centimetres between rows and 15-20 centimetres between plants. A check cultivar was placed every 7-10 rows among the test rows, whereas AC Orford and X5 were placed randomly throughout the field. In addition to the ECORC T_1 plant labels, a plot number was assigned at the GPCRC for field and seed monitoring. Planting was carried out on June 6 and June 25, 1999. Survival for each plant was recorded 20 days after emergence. Standard herbicide treatments, applied at half rates, and manual weeding were used for weed control.

Root evaluations were done during the month of August. All, but six plants in each test row were removed from the soil. A spade that was 14 centimetres wide and 18 centimetres long, was used to make a square cut of approximately 14 x 14 cm and 18 cm deep. The soil cube was lifted into a pail with a plastic bag liner. Water was added to the root pails, and roots were washed with a special low pressure water nozzle. Plants retained approximately 80-90% roots. Roots were qualitatively inspected in the laboratory under a dissecting microscope and scored for SCN resistance as described below.

It was reasoned that each row, which was comprised of T_1 progeny from a transgenic line, should be considered using the rules of Mendelian inheritance. If *Hs1*$^{pro-1}$ or *Hs1*$^{tha-1}$ encoded proteins which conferred SCN resistance in soybean, then it was assumed that the phenotypic expression of these transgenes should behave in a dominant fashion. Thus, among scores for T_1 plants in a row in which a single transgene insertion occurred in the founding primary transgenic plant, we would expect a 3:1 ratio of resistant:susceptible plants. For a double transgene insertion we would expect a 15:1 ratio of resistant:susceptible plants; for a triple, 63:1; and so on. Moreover, a SCN-resistant cultivar is often described as one in which the number of reproductive females or cysts is less than 10 % of the number that develop on a standard susceptible variety (Nieblack 1999). The use of this criterion in our study is complicated by the small stature of AC Orford and X5 cultivars grown under the environmental conditions in southwestern Ontario. Therefore, for a test line to be scored as SCN resistant, it was decided that at least 70% of the plants in the row must have a density of root infection (i.e. cysts/root length) that is less than 10 % of the average density on susceptible plants from the check rows on either side of the test row. T_1 seed from the remaining plants in each of the selected rows was harvested and labelled with their GPCRC plot number plus A, B, C, etc., and saved for future screening.

If enough T_0 seed was available, some lines were screened twice (June 6 and June 25 trials). Root rating results, over both trials if data were available, and field evaluation of morphological size were used for final selection.

4. Antibody development

The carboxyl terminal 172 amino acids of the *Hs1*^{pro1} gene product were expressed as part of a fusion protein employing the entire 26-kDa glutathione S transferase (GST) protein at its amino terminal end. The coding sequence for this chimeric protein was constructed by ligating a *Bam*HI-*Eco*RI fragment from the *Hs1*^{pro1} gene into the expression vector pGEX-2T (Pharmacia; Upsala, Sweden) which was digested with *Bam*HI and *Eco*RI. The resulting plasmid was transformed into *Escherichia coli* strain BL21 and expression of the 52-kDa fusion protein was induced with 0.1 mM IPTG (Sigma, St. Louis, MO). Eight three-litre batches of induced cells were harvested, lysed with lysozyme (Sigma), and the fusion protein was purified by affinity chromatography using glutathione-coated agarose beads (Sigma), followed by electroelution from SDS-PAGE (Sambrook et al., 1989). After dialysis versus water and concentration by freeze drying, the amount of purified protein was estimated, using known standards, by SDS-PAGE. The amount of protein purified from three litres of cells

was low, usually between 50 to 100 µg. Four injections of 20-200 µg protein, with Freund's incomplete adjuvant (Sigma), into one laying chicken were performed by University of Guelph Animal Care Services staff at one- to two-week intervals. Crude IgY was prepared from yolks of eggs collected seven days after the last immunization by mixing yolk with four volumes of water, freezing at -20 ^{0}C for 40 hours, centrifugation at 31,000 xg in a Beckman Ti45 rotor for 30 minutes at 10 C, overnight precipitation of IgY from the supernatant with 50% ammonium sulphate at 4^{0}C, centrifugation at 3,000 x g for 20 minutes at 10 ^{0}C, resuspension of the pellet in 5 ml PBS, and exhaustive dialysis versus PBS at 4 ^{0}C. The sample was then ready for use or freeze dried for storage. This chicken antibody preparation is referred to as chicken anti-*Hs1^{pro1}* antibody (Cαpro1). The Cαpro1 antibody titer was tested by ELISA (Harlow and Lane, 1988) using dilutions of antigen produced in *E. coli*.

Protein from *B. procumbens* apical meristems was prepared by grinding tissue (1 g or more) under liquid nitrogen with a mortar and pestle, suspending the material in four to five volumes of extraction buffer (50 mM Tris pH 7.5, containing 1 mM EDTA, 14 mM ß-mercaptoethanol, 3 % polyvinyl polypyrrolidone, 2.5 µg/ml pepstatin A, 2.5 µg/ml leupeptin and 2.5 µg/ml AEBSF; all reagents were from Sigma, except for AEBSF, which was from BioShop, Burlington, Ontario), and centrifugation at 12,000 x g for 10 min at 4 ^{0}C. The protein concentration of the crude supernatant was determined with the Coomassie Plus Protein Assay Reagent (BioRad, Hercules, CA) before centrifugation. Then the proteins were precipitated with 80 % ammonium sulphate, concentrated by centrifugation, and the protein pellet was dissolved at 3 to 60 µg/µl in extraction buffer. Fifty- to 400-µg protein samples were separated by SDS-PAGE, then transferred to PVDF membrane (BioRad) as a Western blot for immunodetection with the Capro1 antibody. An alkaline phosphatase conjugated rabbit anti-chicken secondary antibody (Sigma) was used to detect the primary Capro1 antibody; development employed Sigma Fast Alkaline Phosphatase detection reagent (Sigma).

D. Results and Discussion

The basic protocol for this project involved four different tasks among three collaborating groups. Molecular plasmid constructions were performed by the group at the University of Guelph. Soybean transformation and regeneration was performed by the group at the ECORC. SCN-resistance assays were performed by the agronomy/nematology group at the GPCRC, at a test site in Ruthven, Ontario. The Guelph group also attempted to produce an antibody for recognition of the *Hs1*$^{pro-1}$ gene product.

1. Plasmid constructions

Five plasmids for soybean transformation were produced (Table 1). Plasmid 52 and 53 were nearly identical constructions, containing the chimeric *ocs/mas* superpromoter followed by the entire *Hs1*$^{pro-1}$ coding sequence plus native terminator and subsequently followed by the CaMV 35S terminator. They differed only in that a unique *Eco*RI site in the *Hs1*$^{pro-1}$ coding sequence was filled-in by the Klenow fragment of DNA polymerase I to create a premature stop codon at position 45, thereby providing a negative control "frame-shift" construction for this project. The 35S terminator was thought to be redundant in these plasmids, so it was removed in the construction of plasmid 79, rendering it functionally identical to plasmid 52. Plasmids 71 and 74 contained *Hs1*$^{tha-1}$ coding and termination sequences, and differed in the promoter sequences which were incorporated; plasmid 71 bore the native *Hs1*$^{tha-1}$ promoter, whereas plasmid 74 bore the superpromoter.

2. Soybean transformations

The ECORC laboratory produced 502 primary transgenic (T_0) plants for this project by particle bombardment transformation and regeneration (Table 1). The *Hs1*$^{pro-1}$ constructions, plasmids 52 and 79 accounted for 57% of these. The negative control, plasmid 53, accounted for 16 %, whereas the *Hs1*$^{tha-1}$ constructions, plasmids 71 and 74, accounted for the rest. Most T_0 plants, 455 of the total, were produced in the X5 soybean host. The remaining 47 T_0 plants were produced in the AC Orford cultivar with plasmid 74.

The *Hs1*$^{pro-1}$ plasmid DNA was extracted from many of the soybean embryos for Southern blot analysis by the ECORC group to establish successful bombardment transformations, and to determine the number of transgenes in the genomes of regenerating T_0 plants. The primary regenerants were then grown to maturity, and again DNA was

extracted from the leaves of many of these for Southern analysis to ensure that transgenics were propagated. Southern blot analyses revealed that regenerated transgenic plants carried one to six transgene copies.

3. SCN-resistance field test

Primary transgenic plants were grown to maturity at the ECORC and T_1 seed were collected and sent to the GPCRC for the field test in the summer of 1999. The initial proposal described a greenhouse SCN resistance bioassay; however, due to the large number of T_0 plants and the much larger number of T_1 generation plants which needed to be screened, a field bioassay was employed.

The numbers of T_1 plants that were in the field and screened in the SCN resistance test are listed in Table 1. Of the 502 T_0 plants produced for this project, T_1 progeny of 334 were screened as individual transgenic lines in rows of up to 20 plants. In total, 4098 T_1 progeny were planted and survived; 68% of these were visually scored for cyst density on the root, and the rest were left in the field to mature and produce seed. To assist in the screening of these transgenic lines, a summer field assistant was employed with funds raised from the Ontario Soybean Growers' Marketing Board.

Table 1. Number of primary transgenic (T_0) plants produced for each of five plasmids employing either the *Hs1*^{pro1} or *Hs1*^{tha1} coding sequences, the number of first generation offspring (T_1) planted and screened for SCN resistance, and the number of lines selected from the field test at Ruthven, Ontario, during the summer of 1999.

Plasmid Number	Promoter/ Coding Sequence/ Terminator	Host	Number of $T_{0\ Plants}$		Number of T_1 Plants		Number of Lines Selected
			Produced	Screened [a]	In Field	Screened [b]	
52	SP [d] *Hs1*$^{pro-1}$ 35S polyA [e]	X5	262	247	2966	2005	15
53	SP *Hs1*$^{pro-1}$FS$_{45}$ [f] 35S polyA	X5	81	22	328	236	3
71	native P [g] *Hs1*$^{tha-1}$ native polyA [h]	X5	12	9	81	32	1
74	SP *Hs1*$^{tha-1}$ native polyA	X5	74	0	0	0	0
		AC Orford	47	47	683	492	7
79	SP *Hs1*$^{pro-1}$ native polyA	X5	26	9	40	24	0
TOTAL			502	334	4098	2789	26

[a] The number of T_0 plants screened were planted as rows of T_1 progeny.

[b] The number of T_1 plants screened was < the number in the field because siblings were left to produce T_1 seed.

[c] Lines were selected if at least 70 % of the plants screened in a row had ≥ cyst density on the roots of less than 10 % of that on check plants.

[d] Superpromoter.

[e] Cauliflower mosaic virus 35S polyadenylylation signal.

[f] Frame-shift (premature translation stop) at *Hs1*$^{pro-1}$ codon 45.

[g] Native *Hs1*$^{tha-1}$ promoter.

[h] Native polyadenylylation signal of respective *Hs1*$^{pro-1}$ or *Hs1*$^{tha-1}$ gene.

Five types of soybean controls were used: Kenwood 94, S20-20, AC Orford, X5 and the T_1 blind negative control lines (i.e. transformants with plasmid number 53). Kenwood 94 and S20-20 are cultivars adapted for the southwestern Ontario soybean growing region and are regarded as susceptible to SCN infections; these were positive controls. The two transgenic plant host cultivars, AC Orford and X5, which are not adapted to this region, did not grow as well as Kenwood 94 and S20-20. Nevertheless, all four cultivars appeared to have similar SCN susceptibilities, as indicated by qualitative assessment of cyst density (not shown). Thus, AC Orford and X5 were suitable positive controls for the assessment of local SCN population density and potential SCN resistance in transgenic plants. The blind controls also provided the means to assess the local SCN population density, as well as to evaluate the experimental field screening process.

Table 2 lists the transgenic lines selected from both field trials. Fifteen lines originated from T_0 plants transformed with the *Hs1*$^{pro-1}$ coding sequence, whereas eight originated from *Hs1*$^{tha-1}$ coding sequence transformations. One of these eight lines, B81 15A2-1, used the native *Hs1*$^{tha-1}$ promoter to drive transcription of the transgene, whereas the other seven used the superpromoter. Three blind control lines are also listed in Table 2, suggesting either that the area in which these plants were grown (i.e. over only seven rows of the field test) has a low SCN population density, or that the validity and accuracy of the screening design is doubtful. At the very least, other lines listed in the table and grown near these blind controls should be considered unlikely to be SCN resistant. Therefore, B73C 7B1-2, which was grown only two rows from the blind control line B73A 6A7-2, should be discarded.

Many lines were planted twice for field screening. Eleven lines expressing Hs1^{pro-1} were selected as SCN-resistant in at least one of the trials (Table 2). Only one line, B73A 9A6-1, was selected in both trials, and it should be considered as high priority for future work towards the development of SCN-resistant lines. Twelve other lines expressing Hs1^{pro-1} or Hs1^{tha-1} were identified as SCN-resistant, but were only screened once. These should be considered lower priority than B73A 9A6-1 until they can be screened again.

4. Antibody development

The Capro1 antibody developed for this project was used to probe two Western blots supporting total apical meristem protein preparations from *B. procumbens*. This tissue was used because abundant *Hs1*$^{pro-1}$ RNA was detected in reverse-transcription polymerase chain reaction (RT-PCR) experiments not reported here. In the first immunodetection experiment,

a single protein band was recognized, although weakly, by the Caprol antibody (not shown). This experiment was repeated with the second Western blot, which also included shoot and root protein samples from wild-type soybean or trangenic soybean transformed with a *Hsl*$^{pro-1}$ plasmid construct. No protein bands of the expected size were revealed in the *B. procumbens* lane, and no bands were revealed in the transgenic soybean lanes which differed from the wild-type soybean lanes (not shown). These results indicated that the *Hsl*$^{pro-1}$ gene product is either expressed at low levels in both *B. procumbens* and transgenic soybean, or that a better antibody preparation is required.

Table 2. Summary of SCN-resistant transgenic lines. Refer to Table 1 for details about transgene plasmid constructions.

Plasmid # / Host	Selected Lines	Trial [1]		Plasmid # / Host	Selected Lines	Trial [1]	
		1ST	2ND			1ST	2ND
52 / X5	B73A 2A6-D	-2	R [3]	52 / X5	B73B 10A2-8	R	S
52 / X5	B73A 2A6-F	S [4]	R	52 / X5	B73C 10A2-1	R	--
52 / X5	B73C 7B1-2	R	--	53 / X5	B73A 6A1-A	R [5]	--
52 / X5	B73A 9A6-1	R	R	53 / X5	B73A 6A6-3	R [5]	--
52 / X5	B73A 9A6-3	S	R	53 / X5	B73A 6A7-2	R [5]	--
52 / X5	B73A 9A6-4	R	S	71 / X5	B81 15A2-1	--	R
52 / X5	B73A 10A1-D	R	S	74 / AC ORFORD	B85 1B5-4	R	--
52 / X5	B73B 10A1-3	R	S	74 / AC ORFORD	B85 1B5-6	R	--
52 / X5	B73B 10A2-0	R	--	74 / AC ORFORD	B85 5A1-11	R	--
52 / X5	B73B 10A2-2	R	S	74 / AC ORFORD	B85 5A1-13	R	--
52 / X5	B73B 10A2-3	R	S	74 / AC ORFORD	B85 5A1-16	R	--
52 / X5	B73B 10A2-4	R	S	74 / AC ORFORD	B85 5A1-19	R	--
52 / X5	B73B 10A2-5	R	S	74 / AC ORFORD	B85 5A3-3	R	--

[1] The first and second trials were June 6 and 25, 1999

[2] "--" indicates that this line was not screened

[3] R indicates SCN resistant (i.e. at least 70 % of the scored number of plants in this line had less than 10 % of the number of cysts as was observed on the susceptible control cultivars)

[4] S indicates susceptible

[5] Indicates that this line was a blind control

E. Conclusions

1. One transgenic line, B73A 9A6-1, produced with the *Hs1^{pro-1}* coding sequence was selected under very high stringency as potentially SCN resistant in two field trials, and therefore has priority for development into a SCN-resistant cultivar. Fourteen other *Hs1^{pro-1}* transgenic lines were also selected as potentially SCN resistant; however, they were only screened once, or performed poorly in one of the two trials. Since the Hs1^{pro-1} antibody is not yet available, it has not been possible to investigate the correlation, if any, between Hs1^{pro-1} protein levels and SCN-resistance.

2 Eight transgenic lines produced with the *Arabidopsis Hs1^{tha-1}* coding sequence were also selected as potentially SCN resistant. Whether these are truly SCN resistant needs to be critically tested, bearing in mind that *A. thaliana* is not resistant to either the root knot or cyst nematodes which infect it.

3. Three blind controls (known to be susceptible) appeared to be resistant in the field screen, leading to uncertainty about how many supposedly resistant lines are actually susceptible, and how much of the laboratory effort that involved soybean transformations was compromised by the qualitative nature of the field screening process.

F. Future Directions

T_1 seed from siblings of line B73A 9A6-1, which were harvested at the end of the field trial, will be screened in a greenhouse for resistance against SCN races 3 and 6. This assay will also allow for plant growth to maturity and collection of T_2 seed. Homozygous lines will be derived from any promising plants selected as SCN resistant, and screened under greenhouse conditions for resistance against all known Ontario SCN races (2, 3, 4 & 6). If resistant lines are obtained, they will also be screened against other pathogenic SCN races.

The blind controls, which were transformed with a silenced *Hs1*$^{pro\text{-}1}$ transgene, should be used more liberally in field assays for SCN resistance assays, as they simultaneously provide indicators of local SCN population density and validity of the screening process. If enough seed is available, these negative controls could be planted in alternating rows among the experimental rows. These controls could also be useful in greenhouse resistance assays; for example, for a common container with several plants, an alternating pattern of control and experimental plants could be used.

Although the *Hs1*$^{tha\text{-}1}$ gene was useful to test as a potential SCN resistance gene, comparisons with the *Hs1*$^{pro\text{-}1}$ gene revealed additional information: (1) the *Hs1*$^{tha\text{-}1}$ gene encodes a predicted polypeptide of 435 amino acids, which is much longer than the published 282 amino acid sequence of the *Hs1*$^{pro\text{-}1}$ gene product; (2) the *Hs1*$^{tha\text{-}1}$ gene has a relatively good start of translation sequence (AAAATGGTT, with the middle triplet being the "start" codon), which conforms well with the consensus plant translational start sequence (ACAATGGCT; Joshi, 1987), whereas the *Hs1*$^{pro\text{-}1}$ gene has a relatively poor translational start (GCTATGAGA); and (3) the predicted amino acid sequence upstream of the ATG start codon in the *Hs1*$^{pro\text{-}1}$ gene reveals approximately 60% sequence homology with that of the *Hs1*$^{tha\text{-}1}$ gene. These comparisons suggested that the *Hs1*$^{pro\text{-}1}$ gene provided by Dr. C. Jung of the Kiel University, Germany, was not full-length, and that sequence at the 5' (upstream) end was missing.

Recently, we determined the missing upstream sequence by RACE-PCR (rapid amplification of cDNA ends using the polymerase chain reaction; Frohman et al.1988). This sequence revealed that the predicted *Hs1*$^{pro\text{-}1}$ protein is comprised of 490 amino acids, and that it possesses an excellent start of translation (data not shown). The full-length coding sequence was placed into a transformation vector by the Guelph lab, and transformation of cultivar Westag was performed by the ECORC lab. Only recently it became possible to

transform Westag, a cultivar adapted to southwestern Ontario; its use should facilitate future testing under field conditions. T_1 seed, which is expected within three to six months, will be used to produce plants for a greenhouse SCN resistance assay. A grant from the Ontario Soybean Growers Marketing Board was acquired to support this project.

For the production of chicken antibodies specific for *Hs1*$^{pro-1}$ protein, we are using a alternative approach, one which has been used successfully at Guelph for three other proteins. Using a number of World Wide Web tools and guidelines, a protocol for prediction and synthesis of antigenic peptides was established. The two peptides chosen were: Cys-Lys-Leu-Ser-Lys-Asp-Lys-Arg-His-Val (Peptide B), and Gln-Ala-Val-Gly-Ser-Gly-Lys-Asp-Cys-Arg-Cys (Peptide B). A mixture of the two peptides was injected into a pair of chickens over a 30-day period and the eggs collected. Currently, the crude IgY fraction from the eggs is being prepared and the antibodies specific to each peptide isolated using affinity chromatography. They will be tested against samples from both *Beta procumbens* and soybean

G. References

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H. Partnerships

This project initiated collaboration among three groups from across Ontario in an effort to produce SCN-resistant transgenic plants. It was now possible to take advantage of new research opportunities; for example, the Guelph and GPCRC groups had not worked with transgenic soybeans, and the ECORC group had not worked on nematode resistance. Valuable lessons regarding future research in these new areas were learned by each group. The fact that a grant from the Ontario Soybean Growers' Marketing Board was sought and awarded to continue this research on SCN resistance using the full-length *Hs1*$^{pro-1}$ gene is testimony to the success of this collaboration.

I. Glossary of terms

AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride HCl
BAC	bacterial artificial chromosome
CaMV	cauliflower mosaic virus
DNA	deoxyribonucleic acid
ECORC	Eastern Cereal and Oilseed Research Centre
ELISA	enzyme linked immunosorbant assay
EDTA	ethylene diamine tetraacetic acid
EST	expressed sequence tag
GST	glutathione S transferase
GPCRC	Greenhouse and Processing Crops Research Centre
IgY	immunoglobulin Y
IPTG	isopropylthio-ß-D-galactoside
PBS	phosphate buffered saline
PVDF	polyvinylidine difluoride
RACE-PCR	rapid amplification of cDNA ends using the polymerase chain reaction
RNA	ribonucleic acid
SCN	soybean cyst nematode
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
UAS	upstream activation sequence